Vaccination against gonadotropin-releasing factor (GnRF) with Bopriva significantly decreases testicular development, serum testosterone levels and physical activity in pubertal bulls

Janett, F ; Gerig, T ; Tschuor, A C ; Amatayakul-Chantler, S ; Walker, J ; Howard, R ; Bollwein, H ; Thun, R

Abstract: The aim of this study was to evaluate the effects of vaccination against gonadotropin-releasing factor (GnRF) on testicular development, testosterone secretion, and physical activity in pubertal bulls. The experiment was performed using 44 bulls aged between 6 and 7 mo. Twenty-three animals were vaccinated twice 4 wk apart with 1 mL of Bopriva (Pfizer, Animal Health, Parkville, Australia) and 21 bulls served as matched controls. Serum GnRF antibody titer and testosterone concentration as well as body weight and scrotal circumference were determined in all bulls for 24 wk from the first vaccination. In addition, physical activity was analyzed in 11 vaccinated and in 10 control animals using the ALPRO DeLaval activity meter system (DeLaval AG, Sursee, Switzerland). The results show that vaccination significantly ($P < 0.05$) influenced all parameters evaluated except body weight. Antibody titers to GnRF began to rise 2 wk after the first vaccination and reached peak values 2 wk after the second injection. Significant group differences in anti-GnRF titer were present for 22 wk following the first vaccination. Testosterone concentrations were significantly lower between weeks 6 to 24 after first vaccination in bulls with Bopriva compared with control animals. In vaccinated bulls testicular development was impaired after the second injection and scrotal circumference was significantly smaller between weeks 8 to 24 after first vaccination. Physical activity of vaccinated bulls was reduced after the booster injection with significant group differences for a continuous period of 106 days. In conclusion, vaccination against GnRF with Bopriva in pubertal bulls decreased testosterone levels in peripheral blood, testicular development, and physical activity but did not affect weight gain.

DOI: https://doi.org/10.1016/j.theriogenology.2012.01.035

Vaccination against gonadotropin-releasing factor (GnRF) with Bopriva® significantly decreases testicular development, serum testosterone levels and physical activity in pubertal bulls

F. Janett[a,*], T. Gerig[a], A.C. Tschuor[b], S. Amatayakul-Chantler[c], J. Walker[c], R. Howard[c], H. Bollwein[a,d], R. Thun[a]

[a] *Clinic of Reproductive Medicine, Vetsuisse-Faculty University of Zürich, Switzerland*

[b] *Cattle practice Tschuor and Gartmann, Ilanz, Switzerland*

[c] *Pfizer VMRD, Melbourne, VICTORIA, Australia*

[d] *Clinic for Cattle, University of Veterinary Medicine Hannover Foundation, Germany*

*Corresponding author. Tel.: +41 44 635 8218; fax: +41 44 635 8942.

E-mail address: fjanett@vetclinics.uzh.ch (F. Janett)

Abstract

The aim of this study was to evaluate the effects of vaccination against gonadotropin-releasing factor (GnRF) on testicular development, testosterone secretion and physical activity in pubertal bulls. The experiment was performed using 44 bulls aged between 6 and 7 months. Twenty-three animals were vaccinated twice 4 weeks apart with 1 mL of Bopriva® (Pfizer Animal Health, Australia) and 21 bulls served as matched controls. Serum GnRF antibody titer and testosterone concentration as well as body weight and scrotal circumference were determined in all bulls for 24 weeks from the first vaccination. In addition, physical activity was analyzed in 11 vaccinated and in 10 control animals using the ALPRO® DeLaval activity meter system (DeLaval AG, Sursee, Switzerland). The results show that vaccination significantly ($P < 0.05$) influenced all parameters evaluated except body weight. Antibody

titers to GnRF began to rise 2 weeks after the first vaccination and reached peak values 2 weeks after the second injection. Significant group differences in anti-GnRF titer were present for 22 weeks following the first vaccination. Testosterone concentrations were significantly lower between weeks 6 to 24 after first vaccination in bulls with Bopriva® compared to control animals. In vaccinated bulls testicular development was impaired after the second injection and scrotal circumference was significantly smaller between weeks 8 to 24 after first vaccination. Physical activity of vaccinated bulls was reduced after the booster injection with significant group differences for a continuous period of 106 days. In conclusion, vaccination against GnRF with Bopriva® in pubertal bulls decreased testosterone levels in peripheral blood, testicular development and physical activity but did not affect weight gain.

Keywords: Behavior; Bull; Castration; GnRF; GnRH; Vaccine

1. Introduction

Immunization against gonadotropin-releasing factor (GnRF) represents an animal-friendly alternative to physical castration in male and female mammals [1,2]. The suppression of reproductive function using an anti-GnRF vaccine has been demonstrated in numerous studies in cattle [3-13], sheep [10,14-21], pig [22-28], horse [29-34] and in wild animals [35-39].

The first commercial available vaccine against GnRF (Vaxstrate®, Peptide Technology Ltd, N.S.W., Australia) was approved in Australia for suppression of estrus in heifers [8]. Because of severe adverse reactions and short duration of effect in the field, Vaxstrate® was withdrawn from the market in 1996. In 1998, Improvac® (Pfizer, Animal Health, Australia) was introduced to the market for immunocastration of male pigs and served as a scientific platform for the development of the horse-specific anti-GnRF vaccine Equity® (Pfizer, Animal Health, Australia). Since 2007, a new vaccine (Bopriva®, Pfizer, Animal Health,

Australia) designed specifically for cattle, has been registered in New Zealand and Australia for the reduction of testosterone blood levels in pubertal bulls. Currently Bopriva® is the only anti-GnRF vaccine available for cattle on the market and has demonstrated good efficacy and safety in a pilot project [13] with young bulls. The latter and several other studies [6,9,20,40,41] have shown that anti-GnRF based vaccines to be efficacious at suppressing testosterone driven sexual and aggressive behavior. In these investigations behavior was only assessed subjectively but no information is available in the literature about behavior changes in vaccinated bulls analyzed objectively by using an electronic activity meter system. Thus, the aim of the present study was to evaluate the effects of the new commercially available cattle specific vaccine Bopriva® on testicular development, testosterone secretion and physical activity in fattening bulls of dairy breeds, housed in small groups and supplied with a high energy diet.

2. Material and methods

2.1. Experimental design

For this experiment a total of 44 dairy bulls calves (Holstein Friesian and Red Holstein n = 21, Brown Swiss n = 23) of one month of age were obtained from different farms. They were housed in groups and fed skim powder-based milk replacer ad libitum with free access to water, hay, straw as well as minerals for 8 weeks until weaning. Thereafter, the animals were kept in an open free stall barn divided into 4 separate pens of 10 to 12 animals each, and a high-energy finishing diet was provided. At an age between 6 and 7 months and a mean (± *SD*) body weight of 228 ± 37.7 kg bulls from 2 pens were allocated to a treatment group (n = 23) and animals from the other 2 pens (n = 21) served as controls. Bulls in the treatment group were vaccinated twice 4 weeks apart with 1 mL Bopriva® (400 µg GnRF-protein conjugate, Pfizer Animal Health, Australia) subcutaneously on the neck. Control animals

received the same amount of saline solution. Treatment effects were evaluated for the duration of 6 months and animals examined every 2 weeks. At each occasion bulls were weighed, scrotal circumference measured (ReliaBull®, Lane Manufacturing Inc., Denver, USA) and blood collected by venipuncture of the Vena coccygealis ventralis using vacutainers (9 mL Z Serum Clot Activator® Vacuette®, Greiner Bio-One GmbH, Kremsmünster, Austria). The blood samples were allowed to clot during 2 *h* at room temperature and after centrifugation (4000 x *g*, 10 *min*) serum was decanted and frozen (-18 °C) until analysis. To monitor physical activity, one pen with vaccinated bulls (n = 11) and one pen with control animals (n = 10) were equipped with an electronic activity measurement system and activity data was recorded from day -3 to day 190 after first vaccination. All animal experimentation was performed following approval from the local Animal Ethics Committee.

2.2. Determination of physical activity

Physical activity was analyzed using the ALPRO® DeLaval activity meter system (DeLaval AG, Sursee, Switzerland). The system was developed to register an increase in activity of cattle approaching heat [42]. It consists of an activity tag (including a mobile ball and an activity-sensing transponder), a remote-antenna, and a receiver connected to a processor interfaced with a computer equipped with ALPRO® DeLaval herd management software (DeLaval AG, Sursee, Switzerland). The activity tag mounted on a collar emits a signal every 14 *sec* if the ball inside the tag is moving (1) or not moving (0). Thus, reliable registration of locomotion and movements of the head of the animal is possible. Signals are monitored, and hourly activity (maximal activity 256) data transmitted via antenna to the receiver/processor, which then sends the data to the computer.

2.3. Hormone analysis and anti-GnRF antibody titers

Anti-GnRF antibody titers

Serum anti-GnRF antibody titers were determined by dissociation enhanced lanthanide fluorescence immunoassay (DELFIA) (Perkin Elmer Pty Ltd, Glen Waverly, Australia). Briefly, 384-well streptavidin coated plates (Perkin Elmer Pty Ltd, Glen Waverly, Australia) were coated for 1 *h* at room temperature with 1 µg/mL biotinylated GnRF peptide in DELFIA buffer (50mM Tris-HCl, 0.9 % NaCl, 0.05 % Tween 20, 20 µM EDTA, 0.2 % ovalbumin). Plates were washed and then incubated with serial dilutions of test cattle serum for 1 *h* at room temperature. Unbound serum and antibodies were removed by washing, and bound antibody was detected by incubating plates for a further 1 *h* with europium labeled protein G (Perkin Elmer Pty Ltd, Glen Waverly, Australia). After washing off excess europium labeled protein G, DELFIA Enhancement Solution (Perkin Elmer Pty Ltd, Glen Waverly, Australia) was added to all wells. After 10 *min* plates were excited at 340 nm and emission at 615 nm measured. Serial dilutions of a standard with a nominal titer of 1/409'600 served as a reference for unknown samples. Non-vaccinated cattle serum served as a negative control.

Testosterone concentrations

Testosterone was determined by enzyme-amplified sensitivity immunoassay (TESTO-EASIA, BioSource Europe S.A, Nivelles, Belgium). The detection limit of the assay was 0.01 ng/mL. All samples were analyzed using a competitive binding assay where a fixed amount of testosterone labeled with horseradish peroxidase (HRP) compete with unlabelled testosterone present in calibrators, controls and samples. Cross reactivity with estrogens and progesterone was 0.023 % and 0.035 %, respectively, with androstenedione 0.76 % and 5-α-dihydrotestosterone 0.61 %. The intra- and inter-assay coefficients of variance were 6.3 % and 8.3 %, respectively.

2.4. Statistical analysis

Statistical analysis was performed using the StatView 5.0 software program (SAS Institut, Dübendorf, Switzerland). A multivariate analysis of variance (ANOVA) with repeated measurements was carried out to assess the effects of group, time of examination and the interaction of group and time on the various parameters. For assessment of bull activity the mean hourly activity per day was considered. Multiple comparisons were performed after Bonferroni adjustment. Non-normally distributed data were log-transformed for this purpose. Values were considered to be statistical significant at $P < 0.05$.

3. Results

3.1. Effects of vaccination

Group, time of examination and the interaction of group and time significantly ($P < 0.05$) influenced all parameters with exception of body weight, which was significantly influenced only by the time of examination.

Anti-GnRF titer

Mean (± *SEM*) serum anti-GnRF antibody titers in bulls with and without Bopriva® for 24 weeks after first vaccination are shown in Figure 1a. Significant differences between groups were apparent from weeks 2 to 24.

Testosterone

Mean (± *SEM*) serum testosterone concentrations in bulls with and without Bopriva® were followed for 24 weeks after first immunization as shown in Figure 1b. Significant differences between groups were noted for weeks 6 to 24.

Scrotal circumference

Mean (± *SEM*) scrotal circumference in bulls with and without Bopriva® were followed for 24 weeks after first immunization as shown in Figure 1c. Significant differences between groups were present at weeks 8 to 24.

Body weight

Mean (± *SEM*) body weight in bulls with and without Bopriva® during 24 weeks after first vaccination is shown in Figure 1d. The weight gain was similar in both groups and no significant ($P > 0.05$) differences were present at any time.

Physical activity

Measurement of activity levels in bulls using the ALPRO® DeLaval meter system provided data showing clear and significant differences between groups. Mean (± *SEM*) hourly activity per day in bulls with and without Bopriva® was followed from day -3 until day 190 after first vaccination as shown in Figure 2. After the booster injection activity was always lower in vaccinated compared to control bulls and significant group differences were noted for a period of 106 days.

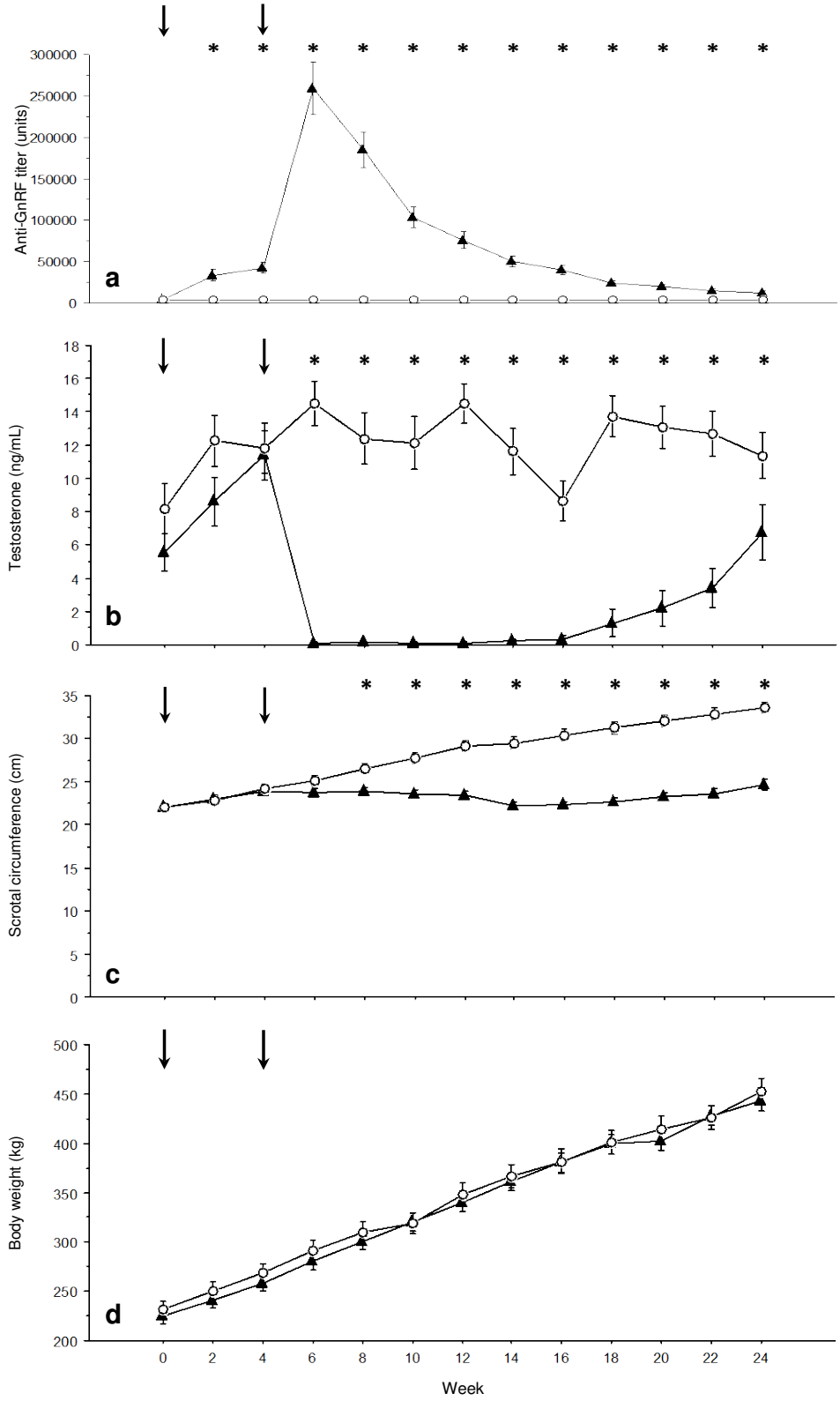

Fig. 1. Means (± *SEM*) of anti-GnRF titer (a), serum testosterone concentration (b), scrotal circumference (c) and body weight (d) in bulls with (▲, n=23) and without (○, n=21) Bopriva®. Arrows indicate injections. *Significant ($P < 0.05$) difference between groups.

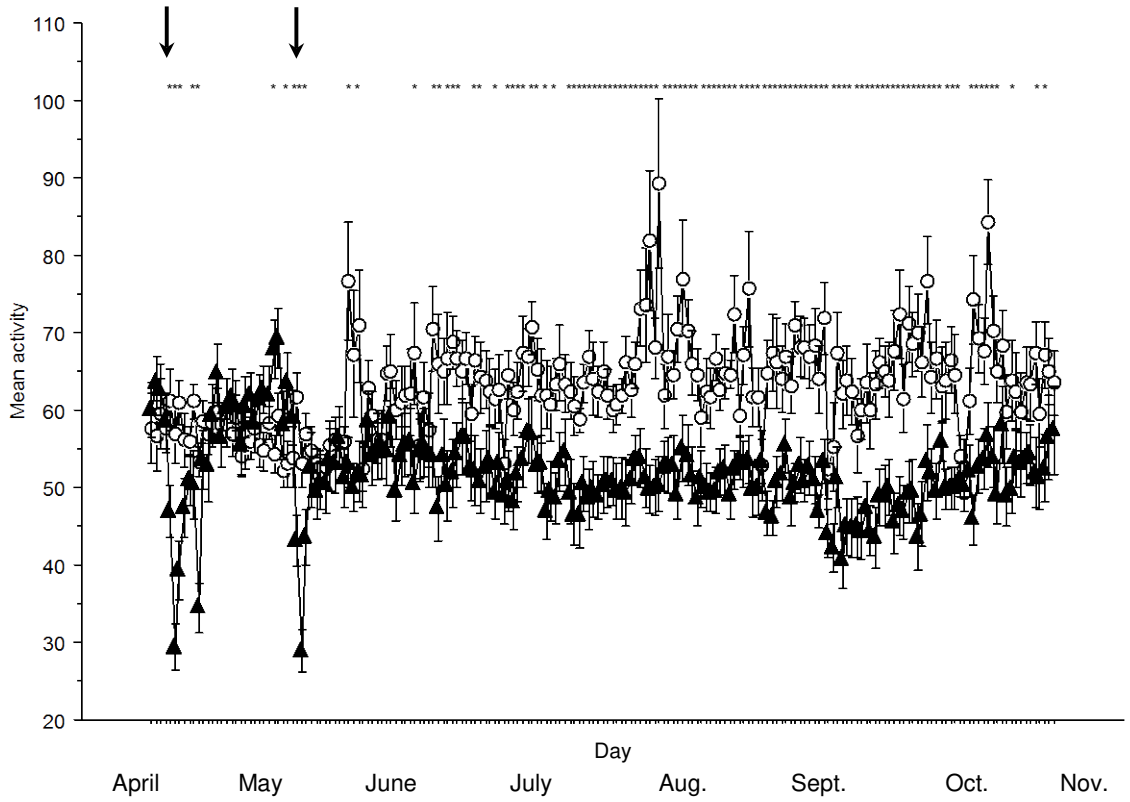

Fig. 2. Mean (± *SEM*) hourly activity per day in bulls with (▲, n=23) and without (○, n=21) Bopriva®. Arrows indicate injections. *Significant ($P < 0.05$) difference between groups.

4. Discussion

This study has shown that vaccination against GnRF with the cattle specific vaccine Bopriva® effectively suppressed testicular development and testosterone secretion in pubertal bulls for at least 12 weeks. Moreover, vaccinated bulls displayed reduced physical activity but similar weight gain when compared to control animals.

As it has been shown in many investigations published during the last years [3-10,17,20,29-34] vaccination against GnRF was able to induce an increase in anti-GnRF antibody titer. When using the specific cattle vaccine Bopriva® as in the present experiment all vaccinated animals showed a strong immune response after the 2 injection. The observed pattern and the

peak values of anti-GnRF titers as well as the suppression of testosterone secretion were comparable to the previous pilot study [13] also using Bopriva® in pubertal bulls aged between 6 and 8 months. When comparing these data with a recent experiment [20] using bull calves vaccinated at an early age of 3 to 6 weeks, mean maximum titer concentration and the duration of testosterone suppression in the older bulls were reduced by approximately half. This suggests that calves as young as 3 weeks are able to produce the same or even a better immune response than older animals, as also reported by Chase et al. [43].

Concomitant with the inhibition of testosterone secretion in vaccinated animals was suppression of testicular growth and scrotal circumference. Testes began to grow again when testosterone secretion started to increase at 12 weeks after the second vaccination. However, the increase in scrotal circumference was less pronounced and remained always lower than in control animals. At the end of the study, 20 weeks after the second vaccination, the difference between vaccinated and control animals was still 9 cm. The observation that vaccination against GnRF resulted in an impairment of testicular development was also found in previous studies in pubertal [4,13] as well as prepubertal [6,20] bulls. In the pilot experiment [13] using Bopriva®, calves were slaughtered 3 months after the booster vaccination and histological examination of testes performed. Vaccinated bulls showed incomplete spermatogenesis with impaired or no production of spermatids and a reduced diameter of the seminiferous tubules. Moreover a recent study [20] demonstrated that all bulls slaughtered 62 weeks after the second vaccination regained spermatogenic capacity reflected by a total sperm count of more than 3×10^9 and sperm motility between 26 and 68 % in semen harvested from the epididymides. From these results it may be concluded that for sustained inhibition of reproduction in bulls revaccination is necessary when testes begin to grow again or testosterone related male behavior starts to be expressed.

Although it is known that testosterone has an anabolic effect, weight gain of vaccinated bulls which had low testosterone levels did not differ from control animals what is consistent with

findings of previous studies [3-6,11,20]. This may be explained by the reduced physical activity in vaccinated animals improving their body condition. Moreover it must be considered, that bulls vaccinated against GnRF have carcass characteristics similar to steers and better feed efficiency than untreated bulls [4] thus increasing economic return to the beef producer.

In this experiment the ALPRO® DeLaval electronic activity meter system has been proven to be very well-suited to detect differences in activity between vaccinated and non-vaccinated bulls. Compared to control animals, the physical activity of bulls treated with Bopriva® was significantly reduced during a total of 106 days after the booster injection. The lowest activities occurred 2 days after both the first and second injection indicating a temporary apathy caused by the vaccination. From previous studies with Bopriva® [13,20] it has been shown that body temperature increased for 1-2 days after both vaccinations without displaying any clinical signs of apathy. The above observations indicate that the ALPRO® DeLaval activity meter system represents a very sensitive tool to detect behavioral changes in bulls and confirm the results of earlier studies where behavior of vaccinated animals was subjectively assessed [6,9,13,20,40,41]. In this regard Jago et al. [9] found that bulls vaccinated with the GnRF vaccine Vaxstrate® showed reduced homosexual mounting, agonistic behavior and damage to pasture, and concluded that immunocastration could provide a practical alternative to traditional methods for bull behavior control.

In conclusion, this study demonstrated that vaccination against GnRF with Bopriva® in pubertal bulls aged between 6 and 7 months led to marked delay in testicular development and to a sustained reduction in physical activity but did not affect weight gain.

Acknowledgements

We would like to thank Hanspeter Renfer, Kaspar Luthiger and Sabine Rinderknecht, Strickhof Eschikon CH-8315 Lindau, for disposal of the barn and the bulls as well as for assistance with animal handling during the experiment.

Conflict of interest

Sue Amatayakul-Chantler, John Walker and Richard Howard are employed by Pfizer Animal Health Australia and acted as Study Monitors. All other authors have no financial or personnel relationship with Pfizer Animal Health and other people or organizations that could influence or bias the study.

References

[1] D'Occhio MJ. Immunological suppression of reproductive functions in male and female mammals. Anim Reprod Sci 1993;33:345-72.

[2] Fayrer-Hosken R. Controlling animal populations using anti-fertility vaccines. Reprod Dom Anim 2008;43:179-85.

[3] Aissat D, Sosa JM, De Avila DM, Bertrand P, Reeves JJ. Endocrine, growth, and carcass characteristics of bulls immunized against luteinizing hormone-releasing hormone fusion proteins. J Anim Sci 2002;80:2209-13.

[4] Cook RB, Popp JD, Kastelic JP, Robbins S, Harland R. The effects of active immunization against GnRH on testicular development, feedlot performance, and carcass characteristics of beef bulls. J Anim Sci 2000;78:27-2783.

[5] D'Occhio MJ, Aspden WJ, Trigg TE. Sustained testicular atrophy in bulls actively immunized against GnRH: potential to control carcase characteristics. Anim Reprod Sci 2001;66:47-58.

[6] Finnerty M, Enright WJ, Roche JF. Testosterone, LH and FSH episodic secretory patterns in GnRH-immunized bulls. J Reprod Fertil 1998;114:85-94.

[7] Geary TW, Grings EE, MacNeil MD, De Avila DM, Reeves JJ. Use of recombinant gonadotropin-releasing hormone antigens for immunosterilization of beef heifers. J Anim Sci 2006;84:343-50.

[8] Hoskinson RM, Rigby RDG, Mattner PE, Huynh VL, D'Occhio MJ, Neish A, Trigg TE, Moss BA, Lindsey MJ, Coleman GD. Vaxstrate: an anti-reproductive vaccine for cattle. Aust J Biotechnol 1990;4:166-70.

[9] Jago JG, Bass JJ, Matthews LR. Evaluation of a vaccine to control bull behaviour. Proc New Zealand Soc Anim Prod 1997;57:91-5.

[10] Jeffcoate IA, Lucas JMS, Crighton DB. Effects of active immunization of ram lambs and bull calves against synthetic luteinizing hormone releasing hormone. Theriogenology 1982;18:65-77.

[11] Neeson D, Colson M. The effects of immuno-castration on bull behaviour and growth rate. Farmer Initiated Technology & Transfer 2004; Final Report - 01FT94.

[12] Stevens JD, Sosa JM, Deavila DM, Oatley JM, Bertrand KP, Gaskins CT, Reeves JJ. Luteinizing hormone-releasing hormone fusion protein vaccines block estrous cycle activity in beef heifers. J Anim Sci 2005;83:152-9.

[13] Theubet G, Thun R, Hilbe M, Janett F. Wirkung einer Impfung gegen GnRH (Bopriva®) beim männlichen pubertären Kalb. Schweiz Arch Tierheilk 2010;152:459-69.

[14] Brown BW, Mattner PE, Carroll PA, Holland E, Paull DR, Hoskinson RM, Rigby RDG. Immunization of sheep against GnRH early in life: effects on reproductive function and hormones in rams. J Reprod Fertil 1994;101:15-21.

[15] Brown BW, Mattner PE, Carroll PA, Hoskinson RM, Rigby RDG. Immunization of sheep against GnRH early in life: effects on reproductive function and hormones in ewes. J Reprod Fertil 1995;103:131-5.

[16] Clarke IJ, Brown BW, Tran VV, Scott J, Fry R, Millar RP, Rao A. Neonatal immunization against gonadotropin-releasing hormone (GnRH) results in diminished GnRH secretion in adulthood. Endocrinology 1998;139:2007-14.

[17] Ferro VA, Khan MAH, McAdam D, Colston A, Aughey E, Mullen AB, Waterston MM, Harvey MJA. Efficacy of an anti-fertility vaccine based on mammalian gonadotrophin releasing hormone (GnRH-I) - a histological comparison in male animals. Vet Immunol Immunopathol 2004;101:73-86.

[18] Janett F, Lanker U, Jörg H, Hässig M, Thun R. Die Kastration männlicher Lämmer mittels Immunisierung gegen GnRH. Schweiz Arch Tierheilk 2003:145;291-9.

[19] Janett F, Lanker U, Jörg H, Meijerink E, Thun R. Unterdrückung der Fortpflanzungsaktivität durch aktive Immunisierung gegen GnRH beim adulten weiblichen Schaf. Schweiz Arch Tierheilk 2009;151:53-9.

[20] Janett F, Gerig T, Tschuor AC, Amatayakul-Chantler S, Walker J, Howard R, Piechotta M, Bollwein H, Thun R. Effect of vaccination against GnRF (Bopriva®) in the prepubertal bull calf. Anim Reprod Sci submitted.

[21] Ülker H, Kanter M, Gökdal Ö, Aygün T, Karakus F, Sakarya ME, Deavila DM, Reeves JJ. Testicular development, ultrasonographic and histological appearance of the testis in ram lambs immunized against recombinant LHRH fusion proteins. Anim Reprod Sci 2005;86:205-19.

[22] Bonneau M, Dufour R, Chouvet C, Roulet C, Meadus W, Squires EJ. The effects of immunization against luteinizing hormone-releasing hormone on performance, sexual development, and levels of boar taint-related compounds in intact male pigs. J Anim Sci 1994;72:14-20.

[23] Clarke I, Walker J, Hennessy D, Kreeger J, Nappier J, Crane J. Inherent food safety of a synthetic gonadotropin-releasing factor (GnRH) vaccine for the control of boar taint in entire male pigs. J Appl Res Vet Med 2008;6:7-14.

[24] Claus R, Lacorn M, Danowski K, Pearce MC, Bauer A. Short-term endocrine and metabolic reactions before and after second immunization against GnRH in boars. Vaccine 2007;25:4689-96.

[25] Dunshea FR, Colantoni C, Howard K, McCauley I, Jackson P, Long KA, Lopaticki S, Nugent EA, Simons JA, Walker J, Hennessy DP. Vaccination of boars with a GnRH vaccine (Improvac) eliminates boar taint and increases growth performance. J Anim Sci 2001;79:2524-35.

[26] Fuchs T, Thun R, Parvizi N, Nathues H, Koehrmann A, Andrews S, Brock F, Klein G, Sudhaus N, Grosse Beilage E. Effect of a gonadotropin-releasing factor vaccine on follicle-stimulating hormone and luteinizing hormone concentrations and on the development of testicles and the expression of boar taint in male pigs. Theriogenology 2009;72:672-80.

[27] Jaros P, Bürgi E, Stärk KDC, Claus R, Hennessy D, Thun R. Effect of active immunization against GnRH on androstenone concentration, growth performance and carcass quality in intact male pigs. Liv Prod Sci 2005;92:31-8.

[28] Zamaratskaia G, Rydhmer L, Andersson HK, Chen G, Lowagie S, Andersson K, Lundström K. Long-term effect of vaccination against gonadotropin-releasing hormone, using Improvac™, on hormonal profile and behaviour of male pigs. Anim Reprod Sci 2008;108:37-48.

[29] Dalin AM, Andresen O, Malmgren L. Immunization against GnRH in mature mares: antibody titres, ovarian function, hormonal levels and oestrous behaviour. J Vet Med 2002;49:125-131.

[30] Elhay M, Newbold A, Britton A, Turley P, Dowsett K, Walker J. Suppression of behavioural and physiological oestrus in the mare by vaccination against GnRH. Aust Vet J 2007;85:39-45.

[31] Imboden I, Janett F, Burger D, Crowe MA, Hässig M, Thun R. Influence of immunization against GnRH on reproductive cyclicity and estrous behavior in the mare. Theriogenology 2006;66:1866-75.

[32] Janett F, Stump R, Burger D, Thun R. Suppression of testicular function and sexual behavior by vaccination against GnRH (Equity™) in the adult stallion. Anim Reprod Sci 2009;115:88-102.

[33] Schanbacher BD, Pratt BR. Response of a cryptorchid stallion to vaccination against luteinising hormone releasing hormone. Vet Rec 1985;116:74-5.

[34] Turkstra JA, Van Der Meer FJUM, Knaap J, Rottier PJM, Teerds KJ, Colenbrander B, Meloen RH. Effects of GnRH immunization in sexually mature pony stallions. Anim Reprod Sci 2005;86:247-59.

[35] Curtis PD, Pooler RL, Richmond ME, Miller LA, Mattfeld GF, Quimby FW. Comparative effects of GnRH and porcine zona pellucida (PZP) immunocontraceptive vaccines for controlling reproduction in white-tailed deer (Odocoileus virginianus). Reprod Suppl 2002;60:131-41.

[36] Killian G, Kreeger TJ, Rhyan J, Fagerstone K, Miller LA. Observations on the use of Gonacon™ in captive Female elk (*Cervus elaphus*). J Wildl Dis 2009;45:184-8.

[37] Massei G, Cowan DP, Coats J, Gladwell F, Miller LA. Effect of the GnRH vaccine GonaCon on the fertility, physiology and behaviour of wild boar. Wildl Res 2008;35:540-7.

[38] Miller LA, Johns BE, Killian GJ. Immunocontraception of white-tailed deer with GnRH vaccine. Am J Reprod Immunol 2000;44:266-74.

[39] Miller LA, Rhyan JC, Drew M. Contraception of bison by GnRH vaccine: a possible means of decreasing transmission of brucellosis in bison. J Wildl Dis 2004;40:725-30.

[40] Price EO, Adams TE, Huxsoll CC, Borgwardt RE. Aggressive behavior is reduced in bulls actively immunized against gonadotropin-releasing hormone. J Anim Sci 2003;81:411-5.

[41] Robertson IS, Wilson JC, Fraser HM. Immunological castration in male cattle. Vet Rec 1979;105:556-7.

[42] Peralta OA, Pearson RE, Nebel RL. Comparison of three estrus detection systems during summer in a large commercial dairy herd. Anim Reprod Sci 2005;87:59-72.

[43] Chase CCL, Hurley DJ, Reber AJ. Neonatal immune development in the calf and its impact on vaccine response. Vet Clin Food Anim 2008;24;87-104.